

ZAP70 Activation Compensates for Loss of Class IA PI3K Isoforms Through Activation of the JAK–STAT3 Pathway

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Abstract. *Background/Aim:* Tyrosine kinases have crucial functions in cell signaling and proliferation. The phosphatidylinositol 3-kinase (PI3K) pathway is frequently deregulated in human cancer and is an essential regulator of cellular proliferation. We aimed to determine which tyrosine kinases contribute to resistance elicited by PI3K silencing and inhibition. *Materials and Methods:* To mimic catalytic inactivation of p110 α / β , specific p110 α (BYL719) and p110 β (KIN193) inhibitors were used in addition to genetic knock-out in *in vitro* assays. Cell viability was assessed using crystal violet staining, whereas cellular transformation ability was analyzed by soft-agar growth assays. *Results:* Activated zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) generated resistance to PI3K inhibition. This resistance was via activation of the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) axis. We demonstrated that activated ZAP70 has a high transforming capability associated with the formation of malignant phenotype in untransformed cells and has the potential to be a tumor-initiating factor in cancer cells. *Conclusion:* ZAP70 may be a potent driver of proliferation and transformation in untransformed cells and is implicated in resistance to PI3K inhibitors in cancer cells. Protein phosphorylation causes activation of signal

transduction pathways, which are crucial for many biological processes (1). The most well-characterized protein kinases in the human genome are protein serine/threonine kinases and protein tyrosine kinases (2). High levels of tyrosine phosphorylation correspond to enhanced proliferative state (3).

The phosphatidylinositol 3-kinase (PI3K) pathway is one of the most activated signaling pathways in human cancer. Upon activation of receptor tyrosine kinases (RTKs), recruitment of PI3K to the cell membrane occurs *via* coupling the Src homology-2 (SH2) domains of p85 with tyrosine phosphorylated residues of activated receptors (4). Oncogenic RAS and other tyrosine kinases can cause PI3K activation. This pathway is also a crucial regulator of cell proliferation, survival and motility (5). Although many findings support the idea that targeting the PI3K pathway is one of the most promising approaches in cancer therapy (4, 6), inhibition of class I PI3K activity by using pharmacological inhibitors leads to retardation of cell proliferation rather than induction of apoptosis, bringing about a problem with generation of drug resistance in the longer run (7).

Previous studies have identified the contribution of several activated tyrosine or serine/threonine kinases to growth compensation upon PI3K inhibition (8-10). In addition to this, pharmacological inhibition of the PI3K pathway in cancer cells leads to up-regulation of receptor tyrosine kinases through feedback loops that circumvent baseline level of PI3K inactivation. These compensatory mechanisms include forkhead box O (FOXO) transcription factors along with mitogen-activated protein kinase (MAPK)-dependent pathways (11, 12). Elucidation of the primary mechanisms of PI3K resistance might be a significant therapeutic approach for tumorigenesis (6). As an example, Pim-1 serine/threonine kinase (PIM1) regulates cell proliferation and survival that might lead to reduction of therapeutic drug efficacy by promoting the Janus kinases/signal transducer and activator of transcription proteins (JAK/STAT) signaling pathway in MYC proto-oncogene (MYC)-driven cancer (13-16), particularly in breast cancer.

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Table I. Activated tyrosine kinase library. The list of activated tyrosine kinase pools is presented. Pool 9, which was investigated in further detail in this study, containing zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) tyrosine kinase, is shown in bold.

Pool #	Gene symbol	Full name
1	<i>ERBB3</i>	erb-b2 receptor tyrosine kinase 3
	<i>ERBB4</i>	erb-b2 receptor tyrosine kinase 4
	<i>FES</i>	FES proto-oncogene, tyrosine kinase
	<i>FGR</i>	FGR proto-oncogene, Src family tyrosine kinase
2	<i>FRK</i>	Fyn-related Src family tyrosine kinase
	<i>HCK</i>	HCK proto-oncogene, Src family tyrosine kinase
	<i>IGF1R</i>	Insulin-like growth factor 1 receptor
	<i>ITK</i>	Interleukin 2 inducible T-cell kinase
3	<i>MATK</i>	Megakaryocyte-associated tyrosine kinase
	<i>NTRK2</i>	Neurotrophic receptor tyrosine kinase 2
	<i>PKMYT1</i>	Protein kinase, membrane associated tyrosine/threonine 1
	<i>TEC</i>	Tec protein tyrosine kinase
4	<i>PTK7</i>	Protein tyrosine kinase 7 (inactive)
	<i>TXK</i>	TXK tyrosine kinase
	<i>ZAP70</i>	Zeta chain of T-cell receptor-associated protein kinase 70
	<i>ERBB2</i>	erb-b2 receptor tyrosine kinase 2
5	<i>MET</i>	MET proto-oncogene, receptor tyrosine kinase
	<i>LCK</i>	LCK proto-oncogene, Src family tyrosine kinase
	<i>NTRK1</i>	Neurotrophic receptor tyrosine kinase 1
	<i>PDGFRA</i>	Platelet-derived growth factor receptor alpha
6	<i>PDGFRB</i>	Platelet-derived growth factor receptor beta
	<i>TNK1</i>	Tyrosine kinase non receptor 1
	<i>FLT3</i>	Fms-related receptor tyrosine kinase 3
	<i>EPHB4</i>	EPH receptor B4
7	<i>DDR2</i>	Discoidin domain receptor tyrosine kinase 2
	<i>MST1R</i>	Macrophage-stimulating 1 receptor
	<i>RET</i>	Ret proto-oncogene
	<i>SYK</i>	Spleen-associated tyrosine kinase
8	<i>TYK2</i>	Tyrosine kinase 2
	<i>ABL</i>	ABL proto-oncogene 2, non-receptor tyrosine kinase
	<i>PTK2</i>	Protein tyrosine kinase 2
	<i>STYK1</i>	Serine/threonine/tyrosine kinase 1
9	<i>FGFR2</i>	Fibroblast growth factor receptor 2
	<i>LYN</i>	LYN proto-oncogene, Src family tyrosine kinase
	<i>PTK2B</i>	Protein tyrosine kinase 2 beta
	<i>PTK6</i>	Protein tyrosine kinase 6
10	<i>ZAP70</i>	Zeta chain of T-cell receptor-associated protein kinase 70
	<i>NTRK3</i>	Neurotrophic receptor tyrosine kinase 3
	<i>DDR1</i>	Discoidin domain receptor tyrosine kinase 1
	<i>EPHA3</i>	EPH receptor A3
11	<i>FLT1</i>	Fms-related receptor tyrosine kinase 1
	<i>MUSK</i>	Muscle-associated receptor tyrosine kinase
	<i>AXL</i>	AXL receptor tyrosine kinase
	<i>LMTK2</i>	Lemur tyrosine kinase 2
12	<i>JAK2</i>	Janus kinase 2
	<i>TYRO3</i>	TYRO3 protein tyrosine kinase
	<i>ALK</i>	ALK receptor tyrosine kinase
	<i>BTK</i>	Bruton tyrosine kinase
13	<i>EPHA4</i>	EPH receptor A4
	<i>EPHA6</i>	EPH receptor A6
	<i>FER</i>	FER tyrosine kinase
	<i>FGFR1</i>	Fibroblast growth factor receptor 1
14	<i>FLT4</i>	Fms-related receptor tyrosine kinase 4
	<i>INSRR</i>	Insulin receptor related receptor
	<i>KDR</i>	Kinase insert domain receptor
	<i>EPHA2</i>	EPH receptor A2
	<i>ROR2</i>	Receptor tyrosine kinase-like orphan receptor 2

Table I. Continued

Table I. *Continued*

Pool #	Gene symbol	Full name
15	<i>CSF1R</i>	Colony-stimulating factor 1 receptor
	<i>EPHA1</i>	EPH receptor A1
	<i>EPHB1</i>	EPH receptor B1
	<i>EPHB6</i>	EPH receptor B6
	<i>JAK1</i>	Janus kinase 1
	<i>FGFR3</i>	Fibroblast growth factor receptor 3
16	<i>JAK3</i>	Janus kinase 3
	<i>SRC</i>	SRC proto-oncogene, non-receptor tyrosine kinase
	<i>EGFR</i>	Epidermal growth factor receptor
	<i>BMX</i>	BMX non-receptor tyrosine kinase
17	<i>FYN</i>	FYN proto-oncogene, Src family tyrosine kinase
	<i>YES1</i>	YES proto-oncogene 1, Src family tyrosine kinase
	<i>ABL1</i>	ABL proto-oncogene 1, non-receptor tyrosine kinase
	<i>BLK</i>	BLK proto-oncogene, Src family tyrosine kinase
	<i>CSK</i>	C-Terminal Src kinase
	<i>TIE1</i>	Tyrosine kinase with immunoglobulin like and interleukin-like domains 1

Zeta chain of T-cell receptor associated protein kinase 70 (ZAP70) is a cytoplasmic tyrosine phosphoprotein. It belongs to spleen tyrosine kinase (SYK) family of nonreceptor tyrosine kinases (17). ZAP70 has a crucial role in T-cell receptor signaling activation and T-cell development (18). Based on structural studies, T-cell activation by antigens results in phosphorylation of LCK proto-oncogene, Src family tyrosine kinase (LCK) and binding of SH2 domains of ZAP70 to tyrosine phosphorylated immunoreceptor tyrosine-based activation motifs which leads to its recruitment to the membrane (19). Existing literature has also described non-immunological functions of ZAP70. ZAP70 was shown to promote migration and invasion of prostate cancer cell lines (20). Moreover, in mammalian oocytes and embryonic stem cells, ZAP70 has a role in maintaining stemness and differentiation by regulating the JAK–STAT3–MYC signaling axis (21). Additionally, ZAP70 was identified as a prognostic marker in colorectal cancer for radiation response (22). In this study, we wanted to identify potential tyrosine kinases that contribute to generating resistance to PI3K inhibition. ZAP70 was identified as a mediator of resistance to PI3K ablation in our genetic screen. We then aimed to understand the potential role of activated ZAP70 in tumor initiation, carcinogenesis and resistance to PI3K inhibition.

Materials and Methods

Generation of cell lines expressing activated tyrosine kinase and cell culture. p110 α ^{flox/flox}; p110 β ^{flox/flox} Mouse embryonic fibroblasts (MEFs) and SV40 large T-antigen-expressing human embryonic kidney 293 (HEK293T) cells, T47D and MCF7 luminal A type breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St Louis, MO, USA)

with 4.5 g/l D-glucose and 1 mM L-glutamine (Sigma–Aldrich) supplemented with 8% fetal bovine serum (FBS, Biowest, Nuaille, France), and 100 IU/ml penicillin and 100 μ g/ml streptomycin. Human telomerase reverse transcriptase-immortalized retinal pigment epithelial-1 (RPE1-hTERT) cells were grown in DMEM/F-12, supplemented with 8% FBS, 1 mM L-glutamine and 100 IU/ml penicillin and 100 μ g/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). The non-tumorigenic human epithelial breast cells MCF10A and human mammary epithelial cells (HMECs) were cultured in DMEM/F-12, supplemented with 4% FBS, penicillin (100 IU/ml), streptomycin (100 μ g/ml), L-glutamine, 10 ng/ml epidermal growth factor (Sigma–Aldrich), 10 μ g/ml insulin (Sigma–Aldrich) and 0.5 μ g/ml hydrocortisone (Sigma–Aldrich). Jurkat cells were maintained in RPMI 1640 medium supplemented with 8% FBS, 1 mM L-glutamine, and 100 IU/ml penicillin/streptomycin. All cell lines were incubated at 37°C with 5% CO₂. The Jurkat and hTERT-RPE1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All the other cell lines have previously been validated and characterized (23).

To identify a tyrosine kinase that potentially contributes to generating resistance to PI3K abrogation, we screened an activated tyrosine kinase library in MEFs which are genetically engineered with LoxP sites, inserted into the first exons of *PIK3CA* and *PIK3CB* (23, 24). The LoxP sequences in these cells can be targeted by Cre recombinase or LacZ-expressing adenoviruses (Ad/Cre, Ad/LacZ; The University of Iowa, Viral Vector Core, Iowa City, IA, USA) for excision of the targeted exons of *PIK3CA* and *PIK3CB* to generate p110 α / β double knock-out cells. The activated tyrosine kinase library has 73 open-reading frames (Table I). This pooled tyrosine kinase library consists of modified receptor and non-receptor tyrosine kinases. The activated tyrosine kinase library constructs have C-terminal dimerization tag; ETS variant transcription factor 6 (ETS6) dimerization domain (TEL) and are tagged with FLAG epitope (25). The tyrosine kinase pools were stably expressed in MEFs and screened using crystal violet growth assay. The TEL-RTK library was stably expressed in cells using the transfection protocol of Lipofectamine 2000 Reagent (Invitrogen,

Carlsbad, CA, USA). To generate HEK293T cells transiently expressing the activated tyrosine kinase library, Lipofectamine 3000 Reagent (Invitrogen) was used. The pWzI green fluorescent protein-expressing (#12269; Addgene, Watertown, MA, USA) and myristoylated AKT serine/threonine kinase 1 (MYR-AKT1)-expressing (#15294; Addgene) MEFs were generated by retroviral transduction. The H-Ras G12V construct (#9051; Addgene) was stably expressed in MEFs and RPE1-hTERT cells using the protocol of Lipofectamine 2000 reagent (Invitrogen).

Cell viability assays and inhibitor treatment. The cells were seeded into 12-well or 6-well plates at a confluency of 30-40% and then treated with increasing concentrations of p110 α -specific inhibitor BYL719 (0.3, 0.5 or 1 μ M, Alpelisib; Selleck Chem, Houston, TX, USA) or p110 β -specific inhibitor KIN193 (1 μ M, AZD6482; Selleck Chem) or with both inhibitors combined (0.3, 0.5 or 1 μ M) for approximately 7-10 days. The inhibitors were supplemented in reduced serum medium (4% FBS) to maintain physiologically relevant levels of growth factors. When control cells reached about 80% confluency, cells were fixed in 10% acetic acid and 10% ethanol for at least 24 hours at room temperature. Cells were then washed with 1 \times non-sterile phosphate-buffered saline and stained with 0.4% crystal violet and 20% ethanol for 1 hour at RT. Cells were washed with distilled water and air-dried. DNA intercalating dye was removed with 1 ml 10% acetic acid. Plates were incubated in destaining solution for 1 hour at room temperature. The optical density of the solution was measured at 595 nm.

Adenovirus/Cre recombinase transduction of MEFs. MEF cell lines were seeded in 6-well plates in duplicates at a density of 1×10^5 cells per well. The day after seeding, cells were treated with Ad/Cre at a multiplicity of infection of 75 in medium containing 2% FBS and incubated for 6-8 h. Then the medium was removed and growth medium was added into cells. The infection was repeated three times to facilitate near-complete excision of the first exons of endogenous *PIK3CA* and *PIK3CB*. The adenovirus/Cre-treated cells were seeded into 12-well plates at a density of 2×10^3 cells per well and cultured for 6 days for cellular viability assay.

Soft-agar assay. The soft-agar assay was performed in 6-well plates as triplicates. To form the bottom layer, 2.5 ml DMEM containing 2.25% low-melting agarose (Sigma-Aldrich) mixture was poured into each well and were solidified at room temperature for at least 30 minutes. Then 2×10^5 cells were resuspended in 9 ml DMEM solution and mixed with 3.2 ml 2.25% low-melting agarose. Two millimeters of this mixture was poured into each well and allowed to solidify. A total of 2-3 ml complete growth medium was added to each well and refreshed every 4-5 days. After 4 weeks, the colonies formed were stained with 0.005% crystal violet and 10% ethanol solution and counted under a microscope.

Antibodies and western blotting. The protein lysates were prepared from MEFs, RPE1-hTERT, MCF10A and HEK293T cell pellets. Then 20-30 μ g of protein was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin (Serva, Heidelberg, Germany) in TBS and then incubated with primary antibodies in TBS with 5% bovine serum albumin and 0.1% Tween. The following primary antibodies were used: PI3K p110 α (C73F8;

Cell Signaling, Danvers, MA, USA), PI3K p110 β (C-8; Santa Cruz Biotechnology, Dallas, TX, USA), phospho-STAT3 (Tyr705) (Cell Signaling), ZAP70 (1E7.2) (Santa Cruz Biotechnology), phospho-ZAP70 (Tyr493/Syk (Tyr526) (Cell Signaling), STAT3 (Cell Signaling), phospho-AKT (Ser473) (Cell Signaling), phospho-AKT (Thr308) (D25E6) (Cell Signaling), phospho-p44/42 extracellular signal-regulated protein kinase 1/2 (ERK1/2) (Thr202/Tyr204) (Cell Signaling), phospho-p70 S6 kinase (Thr389) (Cell Signaling), S6 ribosomal protein (Cell Signaling), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling), β -actin (clone AC74; Sigma-Aldrich) phospho-tyrosine 4G10 (Sigma-Aldrich).

In-silico analysis. Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia2.cancer-pku.cn>) is an interactive platform for analyzing RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from The Cancer Genome Atlas (TCGA) and the Genotype Tissue Expression (GTEx) projects. Analysis of survival was conducted by selecting disease-free survival as the endpoint with a median cutoff of 50% for each gene of interest to generate Kaplan-Meier survival curves. In addition, survival curves were generated using KM plotter by restricting the analysis of progression-free survival by grade and chemotherapy treatment (26). University of Alabama Cancer web portal (UALCAN) database was used to depict the comparison of mRNA expression levels between tumor and normal TCGA datasets (27). The cBio Cancer Genomics Portal was used to indicate the alteration frequency of ZAP70 in various type of cancer using US National Cancer Institute (NCI)-60 cancer cell line studies (28).

Statistical analysis. Two-way analysis of variance and paired *t*-test were used for differential comparison between variables. All statistical analyses were performed in GraphPad Prism 8.0 (San Diego, CA, USA). *p*-Values less than 0.05 were considered significant.

Results

Library screen for identifying factors that generate resistance to PI3K inhibition. Our activated tyrosine kinase library comprises 73 open-reading frames of receptor and non-receptor tyrosine kinases which are activated *via C*-terminal dimerization domain of the TEL transcription factor (Table I) (25). We stably expressed activated tyrosine kinases in MEFs and generated p110 α/β double knock-out cells. As a result of our screening, Pool 9, which included protein tyrosine kinase 2 beta (PTK2B), protein tyrosine kinase 6 (PTK6), ZAP70 and neurotrophic receptor tyrosine kinase 3 (NTRK3), partially restored growth capability upon dual loss of p110 α/β (Figure 1A). In our screen, green fluorescent protein-expressing MEFs were used as a negative control, whereas MYR-AKT1-expressing MEFs served as a positive control as MYR-AKT1 localizes to the cell membrane and becomes constitutively activated (29). Then, we extracted proteins from MEFs that expressed FLAG-tagged activated tyrosine kinases in Pool 9 before and after Ad/Cre treatment and performed western blot analysis. Immunoblotting experiments confirmed the presence of immunoreactive bands compatible with the expected sizes of NTRK3, ZAP70 and PTK6, whereas PTK2B expression was not detected

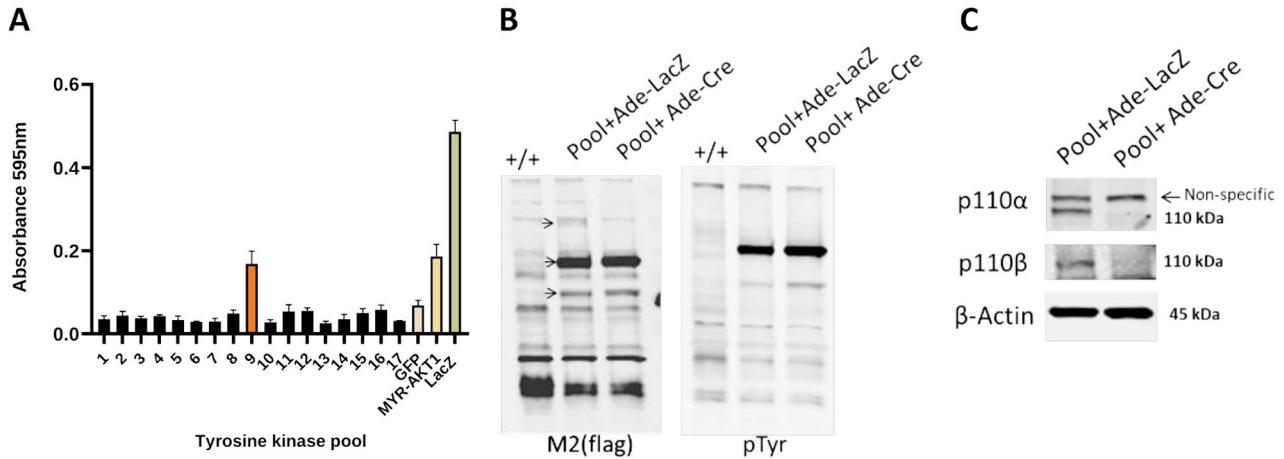


Figure 1. Screening of ETS variant transcription factor 6 dimerization domain (TEL)-tyrosine kinase library. A: The TEL-tagged, activated tyrosine kinases were expressed in mouse embryonic fibroblasts (MEFs). Cells were then treated with Cre-expressing adenoviruses (Ade-Cre). Crystal violet assays were performed and relative cell growth for each pool was calculated. MEFs were transfected with green fluorescent protein (GFP) as a negative control and myristoylated AKT serine/threonine kinase 1 (MYR-AKT1) acted as a positive control. LacZ-expressing MEFs were used as a control for adenoviral infection. The data are the mean \pm standard deviation from experimental triplicates. B: Immunoblot analysis of tyrosine kinase pool 9 including neurotrophic receptor tyrosine kinase 3 (NTRK3), zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70), protein tyrosine kinase 6 (PTK6) and protein tyrosine kinase 2 beta (PTK2B) expression in MEFs by using M2-Flag and phospho-tyrosine primary antibodies (4G10 clone). C: p110 α / β Knockout upon Ade-Cre treatment in pool 9-expressing MEFs. Western blot analysis showed the knockdown efficiency in these cells using primary antibodies for p110 α and p110 β .

(Figure 1B). We also checked for the efficiency of *PIK3CA* and *PIK3CB* knock-out in the cells and found that p110 α and p110 β protein expression was abolished, confirming Ad/Cre targeting (Figure 1C).

TEL-ZAP70 promoted cell proliferation. Cellular growth assays using individual Pool 9 components demonstrated that standalone expression of activated ZAP70 enabled the MEFs to grow efficiently upon Ad/Cre treatment in comparison to those bearing other tyrosine kinases in the pool and to controls (Figure 2A). To understand the role of activated tyrosine kinases in MEFs, we stably transfected these cells with TEL-ZAP70 or TEL-PTK6, using MEFs transfected with MYR-AKT1 as a positive control and pBabe Neo (empty) as a negative control (Figure 2B). We conducted crystal violet cell viability assays. Our results showed that TEL-ZAP70 expression increased the rate of cellular growth to a level similar to MYR-AKT1-expressing MEFs. However, TEL-PTK6 expression in MEFs had no significant effect on cellular growth (Figure 2C).

Investigation of the growth-compensatory potential of activated ZAP70 upon PI3K knockout. In order to confirm the tyrosine kinase library screening, we followed two complementary approaches to inhibit PI3K signaling in MEFs. Firstly, we took advantage of molecular genetics and induced genetic silencing of *PIK3CA* as well as *PIK3CB*, and secondly, we employed pharmacological inhibitors specific for p110 α and - β isoforms. The immunoblot analysis

demonstrated efficient p110 α / β ablation in Ad/Cre-treated MEFs in comparison to controls (Figure 2D). The knockout of p110 α / β caused growth retardation in control MEFs. Nevertheless, TEL-ZAP70 expression in MEFs partially restored the impairment of cellular growth imposed by p110 α / β knockout, as did MYR-AKT1 expression in the positive control (Figure 2E). Of note, the compensation was more pronounced in TEL-ZAP70-expressing MEFs in comparison to MYR-AKT1-expressing MEFs.

For pharmacological inhibition, we used Food and Drug Administration-approved small-molecule inhibitor of PI3K-p110 α , Alpelisib (BYL719), and a specific PI3K-p110 β inhibitor, KIN193 (AZD6482) to selectively inhibit kinase activities of p110 α and p110 β respectively. In general, TEL-ZAP70 MEFs were growth-inhibited to a lesser degree than control MEFs in crystal violet growth assays. We particularly observed a statistically significant reduction of growth inhibition in response to the combination of BYL719 and KIN193 when the cells expressed activated ZAP70 (Figure 2F). These results suggest that activation of ZAP70-mediated signaling induces growth resistance to genetic or pharmacological inhibition of p110 α / β .

TEL-ZAP70 promoted anchorage-independent growth in MEFs. MEFs are untransformed cells which are not capable of growing in an anchorage-independent manner or initiating tumors (30, 31). To determine whether ZAP70 has the ability to transform cells, we performed soft-agar assays with our

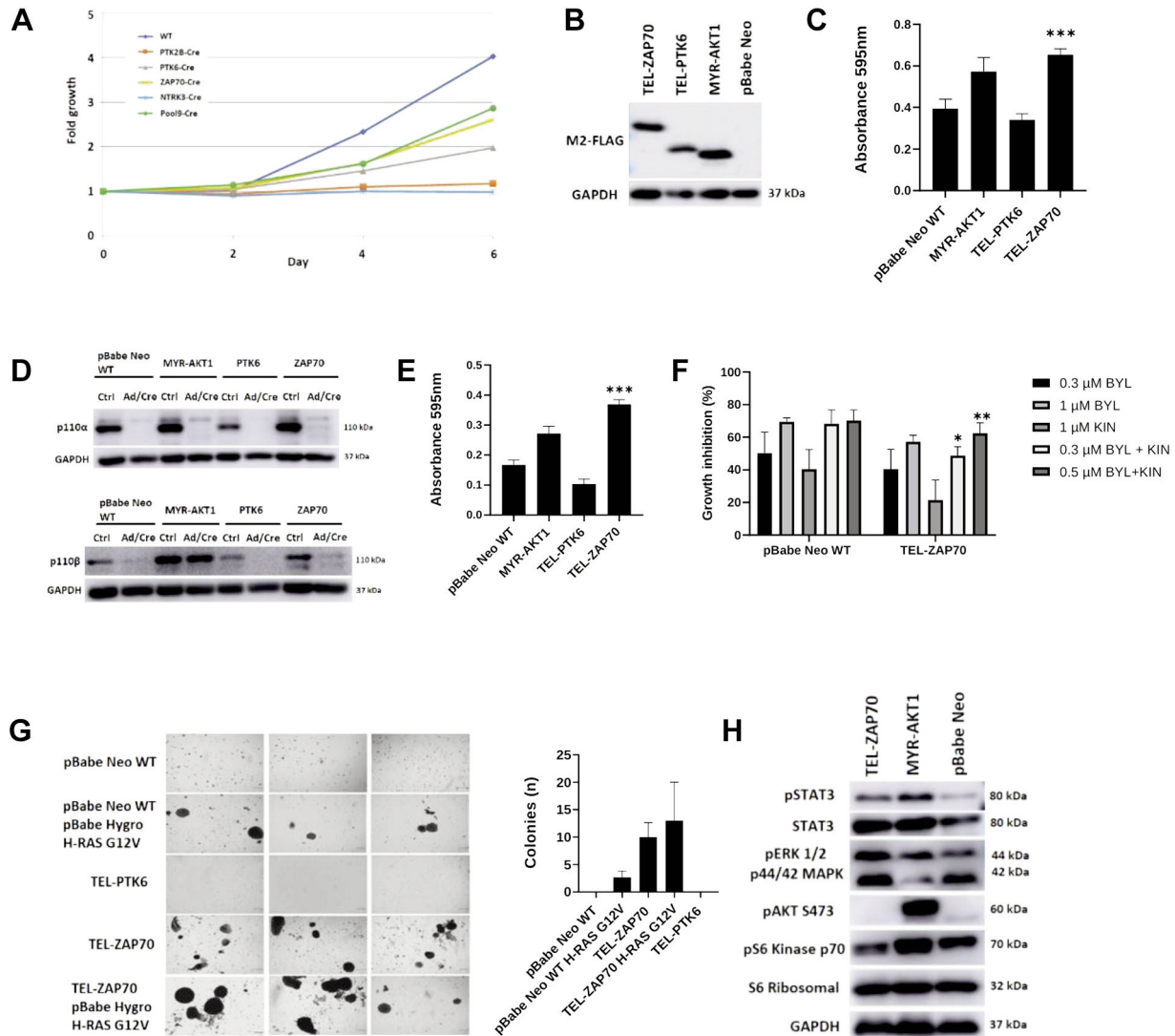


Figure 2. Impact of activated zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) on proliferation of mouse embryonic fibroblasts (MEFs). A: Crystal violet growth assay was conducted for MEFs that express ZAP70, neurotrophic receptor tyrosine kinase 3 (NTRK3), protein tyrosine kinase 6 (PTK6), protein tyrosine kinase 2 beta (PTK2B) and pool 9. Cells were fixed and stained 2, 4 and 6 days after initial seeding. WT: Wild-type. B: Western blot analysis was conducted to confirm the protein expression of individual activated tyrosine kinases in MEFs using M2-FLAG primary antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. C: Crystal violet growth assays showing growth rate of MEFs that expressed activated ZAP70, PTK6, and myristoylated serine/threonine kinase 1 (MYR-AKT1) as positive control and pBabe Neo WT MEFs as negative control. Data shown are the mean±standard deviation (SD), of three independent experiments analyzed by using two-way analysis of variance. ***Significantly different from pBabe Neo WT at $p < 0.0001$. D: Immunoblots showing p110 α/β expression in knockout MEFs. The p110 α/β knockout efficiency was detected using primary antibodies to p110 α and p110 β . E: Crystal violet growth assay was conducted for adenovirus/Cre recombinase (Ad/Cre)-treated MEFs expressing activated ZAP70, PTK6, MYR-AKT1 or pBabe Neo. Data shown are the mean±SD of three independent experiments analysed by using two-way analysis of variance. ***Significantly different from pBabe Neo WT at $p < 0.0001$. F: MEFs expressing activated tyrosine kinase were treated with phosphatidylinositol 3-kinase (PI3K)-p110 α -specific inhibitor Alpelisib (BYL) with/without PI3K-p110 β -specific inhibitor KIN193 (KIN). Crystal violet absorbance at 595 nm was measured. Data shown are the mean±SD of three independent experiments analysed using paired t-test. Significantly different from pBabe Neo WT at: * $p < 0.05$ and ** $p < 0.001$. G: Soft-agar assay was conducted using activated ZAP70 and mutant H-Ras-expressing MEFs. Microscopy images show cells that were able to grow in an anchorage-independent manner. The graphs depict the number of visible colonies in triplicate experiments. Data shown are the mean±standard error of the mean. H: Western blot analysis was performed to detect activated signaling pathways in ZAP70-overexpressing MEFs using antibodies against phospho-specific signal transducer and activator of transcription 3 (pSTAT3-Tyr705), total STAT3, p-S6K p70 (Thr389), total S6, phospho-specific mitogen-activated protein kinase 3/1 (pERK1/2-Thr202/Tyr204), phospho-AKT serine/threonine kinase 1 (p-AKT-Ser473) and MYC proto-oncogene (MYC). MYR-AKT1 expression was used as a positive control for PI3K pathway activation.

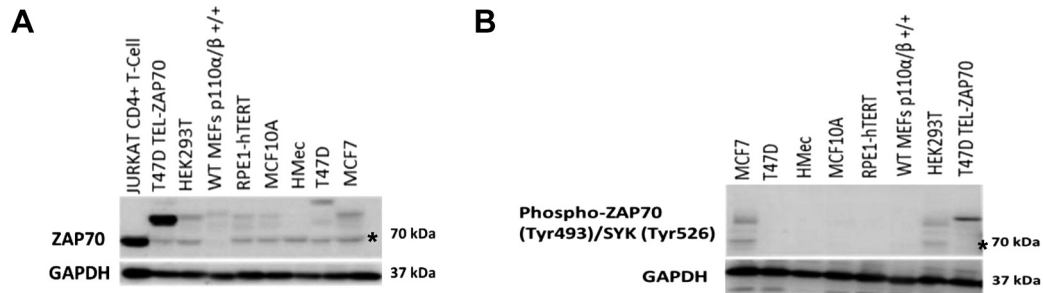


Figure 3. Detection of zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) expression in different cell lines. A: Western blot analysis showing an endogenous ZAP70 expression. The size difference between positive controls was caused by the *ETS* variant transcription factor 6 (*ETV6/TEL*) tag for ZAP70 expression in T47D cells. B: ZAP70 phosphorylation in different cell lines. Asterisks indicate the position of endogenous ZAP70. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

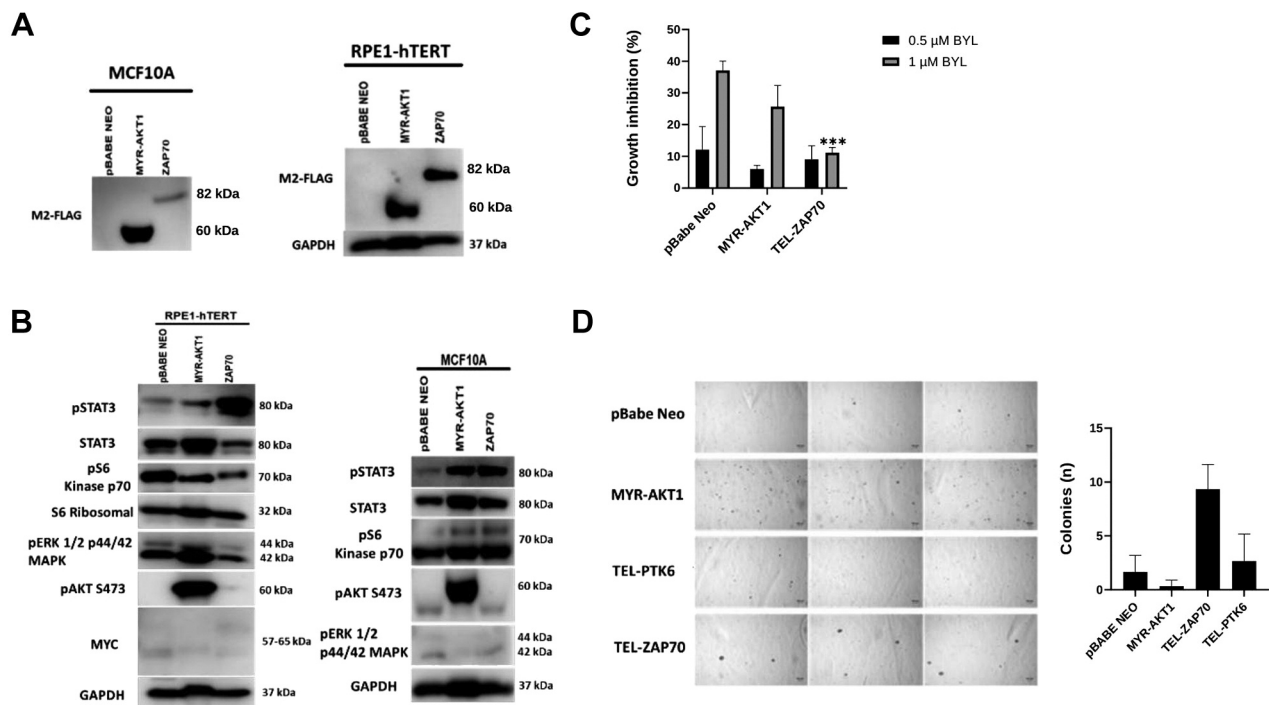


Figure 4. Investigation of the function of zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) expression in non-transformed epithelial cell lines. A: The immunoblots showing *ETS* variant transcription factor 6 (*TEL*)-ZAP70 expression in MCF10A and RPE1-hTERT cells using M2-FLAG antibody. B: Western blot analysis was performed to detect activated signaling pathways with antibodies against phospho-specific signal transducer and activator of transcription 3 (pSTAT3-Tyr705), total STAT3, p-S6K p70 (Thr389), p-S6 (Ser235/236), phospho-specific mitogen-activated protein kinase 3/1 (pERK1/2-Thr202/Tyr204), phospho-AKT serine/threonine kinase 1 (p-AKT-Ser473) and MYC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. C: Crystal violet growth assay analysis showing growth inhibition of depicted RPE1-hTERT lines upon treatment with p110 α - (BYL719) and p110 β (KIN193)-specific inhibitors. Data shown are the mean \pm standard deviation, of triplicate independent experiments and analysed using two-way analysis of variance. **Significantly different from pBabe Neo WT at $p < 0.001$. D: In the same RPE1-hTERT cells, soft-agar growth assays were performed, and representative images are shown on the left. The graph on the right depicts the number of visible colonies. Data shown are the mean \pm standard error of the mean in three independent experiments.

MEF lines that stably expressed activated ZAP70, as well as H-Ras G12V mutant, which constitutively activates H-Ras to enhance transforming ability in mesenchymal cells as a positive control (32, 33).

In this experiment, expression of activated PTK6, which did not compensate for PI3K knockout previously, was used as an additional negative control (Figure 2A). Our results indicated that although activated PTK6- and pBabe

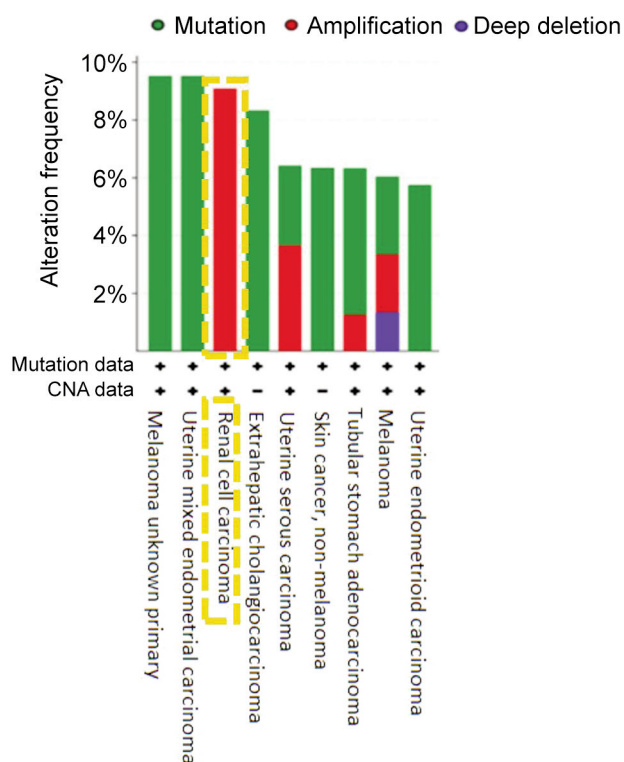


Figure 5. Zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) alterations found in cBioPortal for the US National Cancer Institute (NCI)-60 cancer cell line studies. Based on these studies, ZAP70 amplification occurs highly in renal cell carcinomas. CNS: Copy-number alteration.

Neo empty vector-expressing MEFs were not able to form colonies, ZAP70 overexpression triggered colony formation on soft agar (Figure 2G, right panel). Moreover, co-expression of TEL-ZAP70 and H-Ras G12V had an additive effect on colony formation, implicating a cooperation of ZAP70-mediated signaling with the MAPK pathway (Figure 2G).

ZAP70 expression leads to activation of the JAK/STAT3 pathway. Receptor and non-receptor tyrosine kinases might activate alternative signaling pathways besides PI3K via phosphorylation of downstream targets (34, 35). We hypothesized that the JAK/STAT and MAPK pathways, which have been implicated in functional compensation of PI3K (10, 16), might be activated upon ZAP70-induced partial resistance. To assess the molecular mechanisms that promote compensation of growth upon PI3K knockout in activated ZAP70-expressing MEFs, we conducted western blots to analyze activation of relevant pathways. According to our results, the level of STAT3 phosphorylation was elevated in TEL-ZAP70-expressing MEFs (Figure 2H). In addition, STAT3 phosphorylation was also elevated in MYR-

AKT1-expressing MEFs, which can be explained by the essential role of STAT3 in PI3K-induced oncogenic transformation (36).

Additionally, a slight increase in ERK1/2 phosphorylation was observed (Figure 2H). However, the level of pS6K did not change. The lack of a change in the level of pS6K for ZAP70-expressing MEFs indicates a PI3K-independent mode of growth compensation. Taken together, these observations indicate that growth compensation mediated by activated ZAP70 might occur via phosphorylation/activation of JAK/STAT or MAPK signaling pathways.

To understand if endogenous ZAP70 expression is present in adherent cell lines, we conducted western blot analysis. To this end, Jurkat CD4⁺ T-cells and TEL-ZAP70-expressing T47D cells were used as a positive control. The immunoblot showed that all cell lines, except for p110 α / β ^{+/+} wild-type MEFs, expressed endogenous ZAP70 to various extents (Figure 3A). LCK tyrosine kinase was found to be an important mediator of phosphorylation-dependent activation of ZAP70 (37). To further illustrate if endogenously expressed ZAP70 is phosphorylated and therefore activated, we examined phosphorylation levels of ZAP70. We observed elevated levels of phospho-ZAP70 in TEL-ZAP70-expressing T47D cells, along with HEK293T and MCF7 cells (Figure 3B). Taken together, we detected expression of endogenous and possibly activated ZAP70 expression in several adherent cell lines, including HEK293T, MCF7 and T47D. Several studies showed LCK expression in several solid cancer types [reviewed in (37)], therefore it seems plausible that LCK phosphorylates and activates ZAP70 in a context-dependent manner.

Functional analysis of TEL-ZAP70 expression in alternative, non-transformed epithelial cell culture models. The morphological characteristics of MEFs are different from epithelial cells as they represent cells of mesenchymal origin. Since most carcinomas are derived from epithelial cells, we used non-transformed RPE1-hTERT cells and non-tumorigenic epithelial human breast cells (MCF10A) to understand the impact of activated ZAP70 on initiation of carcinogenesis. We generated cell lines with stable expression of activated ZAP70 along with positive and negative controls in MCF10A and RPE1-hTERT (Figure 4A). Next, we wanted to analyze the activity of ZAP70-mediated signaling pathways in these cells. Our results showed that the phospho-STAT3 level in both cell lines expressing activated ZAP70 was elevated (Figure 4B). Since activated ZAP70 had little effect on pERK and pS6K levels in our study, these results imply that the mechanistic target of rapamycin kinase (mTOR) and MAPK pathways might not be universally affected by ZAP70 activation. However, JAK/STAT signaling pathway appears to be consistently up-regulated by activated ZAP70 in a broad range of cellular models.

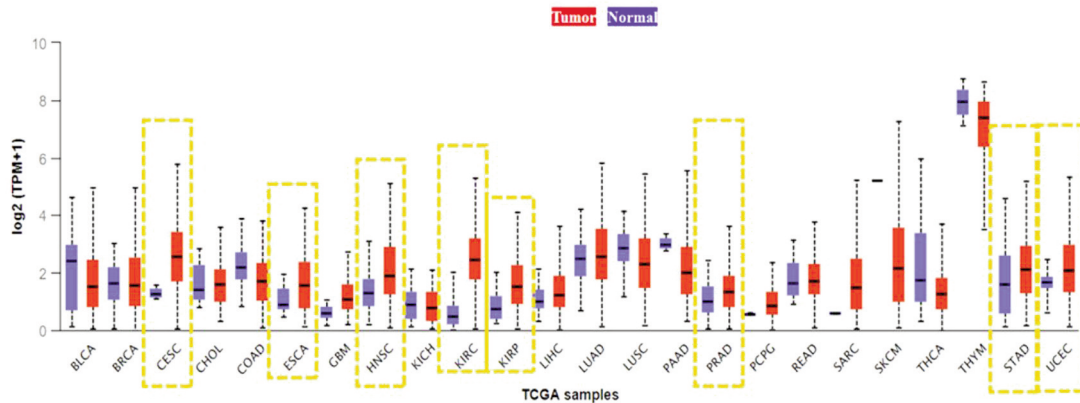


Figure 6. Zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) mRNA expression in tumor and normal tissue in different cancer types from The Cancer Genome Atlas (TCGA). The data were obtained from the University of Alabama Cancer Database (UALCAN). The yellow boxes indicate significantly elevated ZAP70 mRNA expression in solid tumor tissues compared to matched normal tissues. Error bars represent standard deviation. BLCA: Bladder urothelial carcinoma; BRCA: breast invasive carcinoma; COAD: colon adenocarcinoma; ESCA: esophageal carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; HNSC: head and neck squamous carcinoma; KIPAN: kidney chromophobe; KIRC: kidney renal clear cell carcinoma; KIRP: kidney renal papillary cell carcinoma; LIHC: liver hepatocellular carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; PAAD: pancreatic adenocarcinoma; PCPG: pheochromocytoma and paraganglioma; PRAD: prostate adenocarcinoma; READ: rectal adenocarcinoma; SARC: sarcoma; SKCM: skin cutaneous melanoma; STAD: stomach adenocarcinoma; UCEC: uterine corpus endometrial carcinoma; THCA: thyroid carcinoma; THYM: thymoma.

We next assessed whether activated ZAP70 had a positive effect on cell viability upon PI3K inhibition in RPE1-hTERT cells. We used TEL-ZAP70-expressing RPE1 cells along with controls (pBabe Neo and MYR-AKT1-expressing RPE1 cells). Crystal violet growth assays demonstrated that TEL-ZAP70 expression in RPE1-hTERT cells partially compensated growth upon BYL719-mediated inhibition of p110 α (Figure 4C).

After determining the importance of activated ZAP70 for cellular proliferation in RPE1-hTERT cells, we wanted to see if TEL-ZAP70 had the ability to form colonies in anchorage-independent growth assays. We performed soft-agar growth assays and found that TEL-ZAP70-expressing RPE1-hTERT cells were able to form colonies (Figure 4D), although constitutively activated AKT was unable to induce colony formation. Taken together, expression of activated ZAP70 in non-transformed epithelial cells can increase their proliferative potential as well as their ability to transform into more malignant phenotypes.

Analyzing the involvement of ZAP70 in solid tumors in silico. ZAP70 activation has been associated with leukemia (38). We performed *in silico* analyses to determine if it might also be involved in initiation or progression of solid tumors. Analyses of the US National Cancer Institute's NCI-60 cell lines studied using the cBioPortal platform revealed that, ZAP70 is moderately altered in renal cell carcinomas (9%), and these alterations mostly occur in the form of gene amplification (Figure 5).

Moreover, we investigated ZAP70 mRNA expression changes in tumor vs normal tissue for solid tumors. For this purpose, we used the University of Alabama cancer web portal in UALCAN and found that in many cancer types, such as renal clear-cell carcinoma, renal papillary-cell carcinoma, cervical squamous cell carcinoma, endocervical adenocarcinoma, esophageal carcinoma, prostate adenocarcinoma, stomach adenocarcinoma, uterine corpus endometrial carcinoma, as well as head and neck squamous carcinoma, ZAP70 mRNA levels were found to be higher in tumor tissues in comparison to matched controls (Figure 6).

We wished to expand our observations to other types of cancer. To this end, we performed overall survival analysis in various cancer types. The Kaplan–Meier plots showed that low expression of ZAP70 in gastric cancer correlated with better survival (Figure 7A). Additionally, the Kaplan–Meier curves generated using the GEPIA2 platform demonstrated that low ZAP70 expression correlated with better survival in renal clear-cell carcinoma, low-grade glioma, and uveal melanoma (Figure 7B–D). Next, to delineate whether ZAP70 has a differential effect on distinct stages of tumorigenesis, we plotted stage-specific Kaplan–Meier survival curves. Our results showed that low expression of ZAP70 in stage 2 and stage 3 gastric cancer correlated with better survival (Figure 8A and B). These *in silico* analyses support our findings implying a causative role for ZAP70 in various types of solid tumor. To further investigate involvement of ZAP70 in solid tumors, we plotted Kaplan–Meier survival curves in ovarian cancer using KM Plotter database. In survival analysis of patients with grade 1-2 ovarian cancer treated with

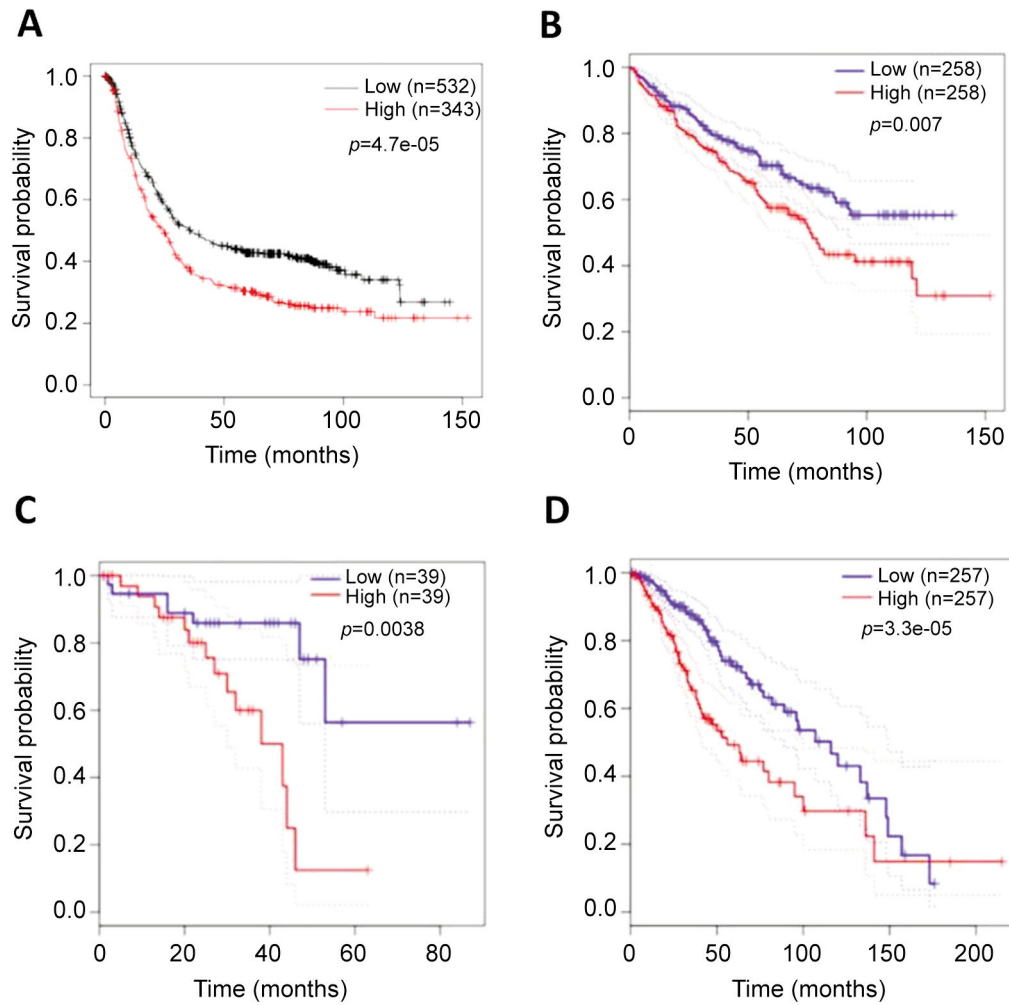


Figure 7. Kaplan–Meier graphs depicting overall survival according to expression of zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) in patients with gastric cancer using KM Plotter database for mRNA gene chip data (2104032_at) (A) and with renal clear-cell carcinoma (B), uveal melanoma (C) and low-grade glioma (D) via GEPIA2.

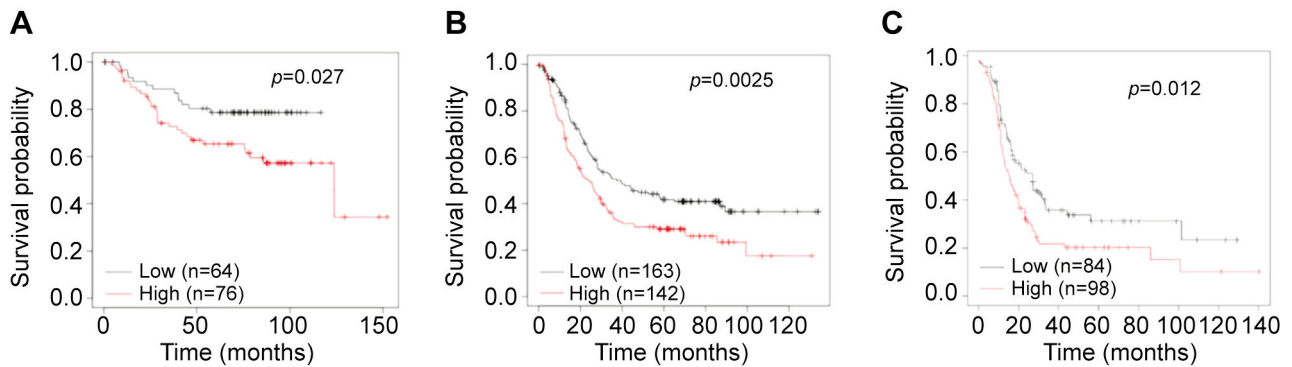


Figure 8. Kaplan–Meier survival graphs depicting survival according to expression of zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) in patients with gastric and ovarian cancer using KM Plotter database for mRNA gene chip data (2104032_at). Overall survival of patients with stage 2 (A) and stage 3 (B) gastric cancer. Progression-free survival of patients with grade 1+2 ovarian cancer treated with paclitaxel/cisplatin (C).

paclitaxel/cisplatin chemotherapy, high ZAP70 expression levels significantly correlated with reduced progression-free survival (Figure 8C). In conclusion, ZAP70 might be a crucial prognostic marker for ovarian carcinogenesis and low ZAP70 expression correlated with better survival in patients treated with chemotherapy for early-stage ovarian cancer.

The role of ZAP70 overexpression in kidney cell lines. According to our *in-silico* analysis, renal cell carcinomas had the highest frequency of ZAP70 amplifications (Figure 5). We wanted to investigate the impact of ZAP70 overexpression in kidney-derived cell lines to consolidate our findings in previous cellular models. To that end, we used embryonic kidney-derived HEK293T cells and transiently expressed activated ZAP70 along with MYR-AKT1 as a positive control and the empty vector (pBabe Neo). Expression of TEL-ZAP70 dramatically enhanced STAT3 phosphorylation in HEK293T cells. However, levels of phospho-S6 kinase and phospho-ERK along with c-MYC did not change (Figure 9). These results imply that ZAP70 activity triggers activation of the JAK/STAT signaling pathway in epithelial as well as in mesenchymal cellular models of tumorigenesis. In conclusion, activated ZAP70 functions as a novel transforming factor in solid tumors and induces partial resistance to PI3K inhibition via phosphorylation and activation of STAT3 in the JAK/STAT signaling axis.

Discussion

In this study, we screened an activated tyrosine kinase library and identified ZAP70 as mediator of resistance upon simultaneous loss of p110 α / p110 β in MEFs (Figure 1). ZAP70 plays critical roles in T-cell/B-cell development and T-cell receptor signaling as well as being involved in hematological malignancies (38). Our results describe a potentially novel role for ZAP70 in untransformed cell lines besides its immunological functions. Our cell viability assays showed that TEL-ZAP70 expression in MEFs significantly enhanced cellular proliferation in comparison to negative control pBABE NEO and positive control MYR-AKT1 (Figure 2). These analyses indicate that active ZAP70 can promote cellular proliferation in adherent cells.

Existing literature supports the notion that PI3K is a crucial regulator of cellular proliferation, and its impairment has been linked to severe growth defects in various cancer models (3, 7). In order to abrogate PI3K signaling, we knocked out endogenous p110 α /p110 β in MEFs. For this, we utilized genetically engineered MEFs, where p110 α and p110 β can be effectively knocked out by expression of Ad-Cre. Although, active PTK6 expression was not able to restore growth in the p110 α /p110 β knockout system (Figure 2), TEL-ZAP70 expressing MEFs largely recovered growth upon p110 α /p110 β ablation. To abolish the catalytic activity

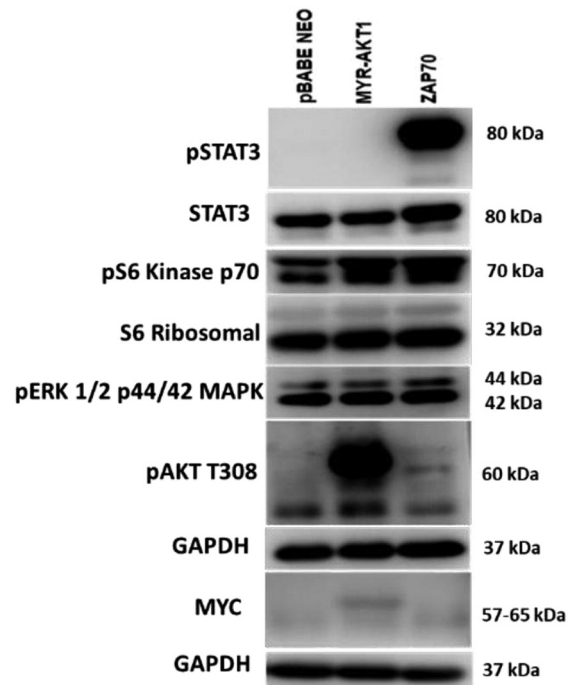


Figure 9. Zeta chain of T-cell receptor-associated protein kinase 70 (ZAP70) overexpression in HEK293T cells. Western blot analysis was performed to detect ETS variant transcription factor 6 (TEL)-ZAP70-mediated activation of downstream signaling components with antibodies against phospho-specific signal transducer and activator of transcription 3 (pSTAT3-Tyr705), total STAT3, p-S6K p70 (Thr389), p-S6 (Ser235/236), phospho-specific mitogen-activated protein kinase 3/1 (p-ERK1/2-Thr202/Tyr204), phospho-AKT serine/threonine kinase 1 (p-AKT-Thr308) and MYC proto-oncogene (MYC). Glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. MYR-AKT1 was used as positive control for phosphatidylinositol 3-kinase pathway activation.

of PI3K, we used clinically relevant p110 α -specific Alpelisib and p110 β -specific AZD6482 pharmacological inhibitors. Our results show that active ZAP70 expression can induce partial resistance to PI3K inhibition in various cellular models comprising both epithelial as well as mesenchymal cells (Figure 2 and Figure 4).

As proposed by Siveen *et al.*, STAT3 can be activated by Src family kinases (39). Furthermore, Warmuth *et al.* suggested that the STAT3 signaling cascade is crucial in carcinogenesis (40). We performed western blot analysis to elucidate the mechanism of TEL-ZAP70-mediated activation of downstream pathway signaling upon PI3K ablation. Our results indicate that up-regulation of STAT3 phosphorylation on tyrosine 705 residue is a ubiquitous event in active ZAP70-expressing cell lines including RPE1-hTERT, MEFs, non-tumorigenic epithelial breast (MCF10A) and human embryonic kidney cells (HEK293T) cell lines (Figure 2,

Figure 4 and Figure 9). Remarkably, p-ERK1/2 and p-S6 kinase p70 expression levels did not change in TEL-ZAP70 RPE1 cells. Additionally, dual-tyrosine phosphorylation of ERK in TEL-ZAP70 MEFs (Figure 2) and phosphorylation of S6K p70 levels in TEL-ZAP70 HEK293T (Figure 9) only slightly increased. Cha *et al.* suggested that the JAK/STAT3/c-MYC signaling axis is negatively regulated to modulate differentiation capability by ZAP70 in mouse embryonic stem cells (21). In contrast to their findings, our results suggested that STAT3 activation was positively correlated with ZAP70 expression (21). In addition, we found out that the c-MYC levels were not altered upon expression of active ZAP70 in TEL-ZAP70 expressing cells (Figure 4 and Figure 9). These contrasting observations might be explained by different genetic contexts in pluripotent stem cells *versus* differentiated or transformed mesenchymal/epithelial cell lines.

To further explore the function of ZAP70 in carcinogenesis, we investigated involvement of ZAP70 in anchorage-independent growth on soft agar in untransformed cell lines. The morphological characteristics of MEFs are different from epithelial cells as they represent cells of mesenchymal origin, whereas most carcinomas are of epithelial origin. Thus, we used non-transformed epithelial cells, MCF10A and RPE1-hTERT, to understand the impact of activated ZAP70 on initiation of carcinogenesis. We found that ZAP70-expressing MEF and RPE1 cells were capable of forming colonies on soft agar (Figure 2 and Figure 4). We also showed that ZAP70 had an additive effect in anchorage-independent growth along with H-Ras G12V (Figure 2). These findings imply that ZAP70 might have a function in tumor initiation. Although our research was mainly conducted on untransformed cell lines, it would be important to decipher the effects of ZAP70 in transformed cancer cells. Based on the literature, ZAP70 promotes cell migration and invasion of prostate cancer cell lines (20). Moreover, ZAP70 was identified as a prognostic marker in cervical squamous cell carcinoma (41), prostate adenocarcinoma (42) and colorectal cancer in response to radiation (22). Furthermore, Sun *et al.* indicated that ZAP70 may be a crucial regulator of metastasis in prostate cancer (42). Of note, Sadras *et al.* found that while ZAP70 has an oncogenic role, SYK, a member belonging to the same family, functions as a tumor suppressor in autoimmune diseases and B-cell malignancies (43).

According to our *in-silico* analysis, ZAP70 mRNA expression levels were found to be elevated in many solid tumor tissues in comparison to matched controls (Figure 6). Additionally, the Kaplan–Meier plots show that low ZAP70 expression in several cancer types, including gastric cancer, uveal melanoma, kidney clear cell carcinoma and low-grade glioma, was associated with better survival (Figure 7). Moreover, our survival analysis depicted that in patients with low-grade ovarian cancer who were treated with paclitaxel and

cisplatin, low ZAP70 expression correlated with better survival (Figure 8). Consequently, high ZAP70 expression might have a tumorigenic/oncogenic function during the establishment of the disease and correlate with a worse prognosis.

In conclusion, our results implicate ZAP70 in tumor initiation as well as in resistance to PI3K inhibition. Although ZAP70 expression has been implicated in poor prognosis in hematological malignancies such as chronic lymphocytic leukemia (44), our results suggest that ZAP70 tyrosine kinase activation can promote cell proliferation and contribute to generation of resistance to PI3K inhibition. The induction of resistance to PI3K inhibition might be explained through activation of JAK/STAT; in particular, the JAK/STAT3 signaling axis. We believe that these findings are of strong clinical relevance as several PI3K inhibitors have already passed clinical trials and are being used in the clinic (45, 46).

Conflicts of Interest

The Authors declare no competing conflicts of interest.

Authors' Contributions

Onur Cizmecioglu: Conception and design, analysis and interpretation of data, and revision of the article. Melike Demir: Acquisition of data, analysis and interpretation of data, and drafting of the article.

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